SUPPORTING INFORMATION

Semiquantitative Nucleic Acid Test with Simultaneous Isotachophoretic Extraction and Amplification

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Table of contents

This Supporting Information document describes:

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Initial locations of sample and analyte

In this study, we use three distinct configurations for the initial locations of sample and analyte. In Figure S-1 we provide schematics of the glass fiber strip in order to clearly illustrate where serum, whole blood, nucleic acid analyte, and ITP buffers are disposed into the membrane before application of a voltage bias. We demonstrate nucleic acid amplification in an ITP plug with the target in pure LE buffer. In this case, ITP is focusing the target DNA and RPA reagents into a concentrated plug, but not significantly extracting the target DNA from a complex sample or the sample pad. As shown in Figure S-1A, an LE solution mixed with RPA reagents (*i.e.* proteins, enzymes, primers, probe, and additives) fills the majority of the strip, including the square sample region of the strip. TE buffer wets the portion of the strip near the TE reservoir. Target DNA (sequence listed in Table S-1) is spiked directly to the center of the strip. We then performed simultaneous ITP-RPA to extract and amplify target DNA from a 20 µL serum sample. Figure S-1B shows, prior to application of an electric field, serum and target DNA are located in the square sample region while LE and RPA reagents fill the majority of the glass fiber strip. Finally, we added unprocessed whole blood to a separation membrane to fill the sample region of the glass fiber membrane with plasma and target DNA. Figure S-1C shows a cutout of the separation membrane with red and white blood cells while filtered plasma wicks below into the glass fiber.

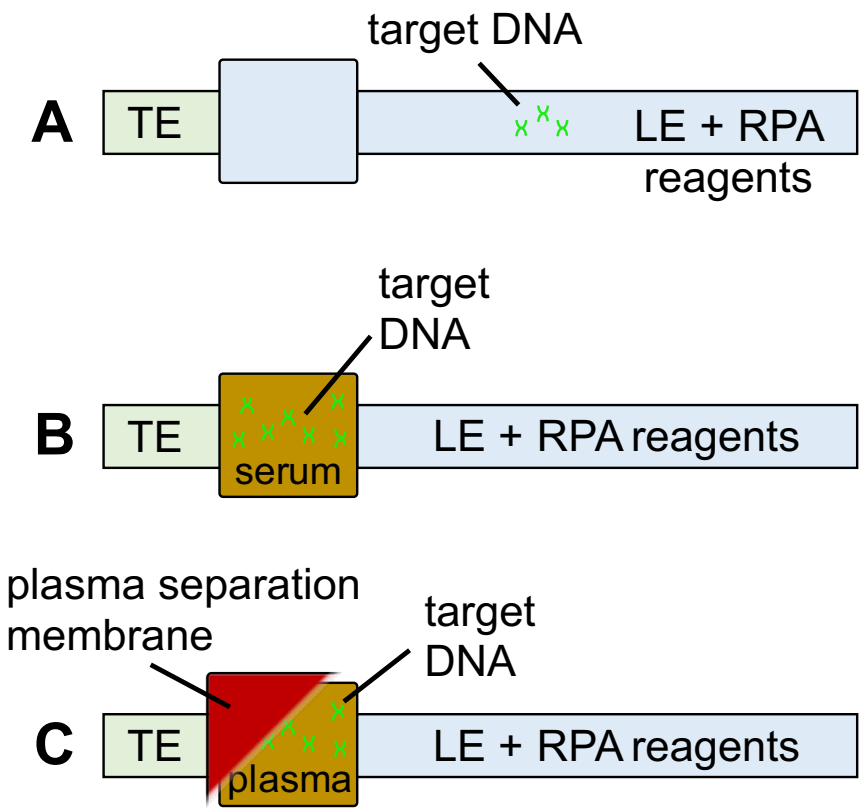

Figure S-1. Schematics of varied configurations of solutions and samples. (A) Simultaneous ITP-RPA experiments in pure buffer utilize an LE solution mixed with RPA reagents. This solution fills the majority of the strip, excluding a small region by the TE reservoir. Target DNA is located in the center of the strip prior to ITP initiation. (B) Simultaneous ITP-RPA experiment from human serum also uses LE solution with RPA reagents. 20 µL of serum with target DNA in the sample region separates the two ITP buffers. (C) ITP-RPA with a 50 µL whole blood sample uses an in-line plasma separation membrane (shown as a cutout) to remove red and white blood cells while plasma and target fills the glass fiber sample pad below. The LE and amplification reagents solution as well as the TE solution are applied as before.

Nucleic acid sequences

Table S-1 lists the sequences of the dsDNA analyte in addition to a forward primer, reverse primer, and sequence-specific probe. The RPA assay is a slightly modified version of an HIV-1 assay developed by Boyle *et al.*[1] The 200 bp DNA target was adopted from a region of the HIV-1 *pol*

gene. Forward and reverse primers were slightly modified to hybridize with the target without mismatches.

Table S-1. Sequences of the target DNA, primers, and sequence specific probe for the RPA assay, as well as the labeled DNA used for extraction-only experiments. bp = nucleotide base pair; FAM = fluorescein amidite; dR = an abasic residue with a fluorophore attached to the ribose via a C-O-C linker; [dSpacer] = abasic site; [T(BHQ-1)] = Black Hole Quencher-1 bound to an internal Thymine residue; Spacer C3 = a moiety that replaces the 3'-OH group preventing nucleotide extension from an intact probe.

Description	**Sequence (5' – 3')**
Abridged HIV-1 *pol* dsDNA (200bp)	AGGCTGAACATCTTAGGACAGCAGTACAAATGGCAGTATTCATTCAC AATTTTAAAAGAAAAGGGGGGATTGGGGGGTACAGAGCAGGGGAAA GAATAGTAGACATAATAGCAACAGACATACAAACTAGAGAACTAGGTT GAGAAATTAGAAAAGTTGAATATGTTAGGGTTTATTACAGGGACAGCA GAGATCCACTT
Forward primer (34bp)	TGGCAGTATTCATTCACAATTTTAAAAGAAAAGG
Reverse primer (34bp)	CCCTAACATATTCAACTTTTCTAATTTCTCAACC
Probe (48bp)	TGCTATTATGTCTACTATTCTTTCCCC[T(FAM)]GC-[dSpacer]C-[T(BHQ 1)]GTACCCCCCAATCCCC-SpacerC3

Per-test cost estimate

Table S-2 provides a per-test cost estimate for our ITP-RPA assay. Devices for performing simultaneous ITP-RPA were constructed with low-cost materials, such as petri dishes, glass fiber, and titanium electrodes. ITP buffers consist of common chemical compounds that can be bought in bulk, resulting in low per-test costs. Currently, RPA reagents can only be purchased through the developer, TwistDx Limited, and resulted in the largest per-test expense. We expect that further development of the test may replace several components with less expensive ones that can be manufactured at scale. For example, the Petri dish and reservoirs may be replaced by a single injection modeled part. We also expect that reagents purchased in bulk or using other amplification chemistries can dramatically reduce the cost of goods.

Table S-2. Estimated costs for different components of the disposable device for ITP-RPA. The provided material costs are based on the list price for each item purchased through respective vendors.

Material Description	Cost
Petri dish	$ 0.10
Acrylic reservoirs	$ 0.05
Glass fiber	$ 0.01
Vivid membrane	$ 0.07
Titanium electrodes	$ 0.06
RPA reagents	$ 2.40
ITP buffers	$ 0.20
Total	**$ 2.89**

Translation of RPA assay to a porous membrane

We created a custom buffer and modified reaction conditions in order to translate our RPA assay from the traditional tube-based format to *in situ* amplification within a membrane. In their study of *in situ* isothermal amplification, Linnes *et al.* described reaction inhibition due to select membranes employed in the diagnostics field.[2] After a membrane screening, we found that glass fiber (Millipore) blocked with 1% w/v BSA and washed with 0.01% v/v Triton-X 100 was a suitable porous matrix for RPA. The exact formula of TwistAmp exo is proprietary, yet the fundamental RPA publication from Piepenburg *et al.* reported an RPA assay in a Tris-acetate buffer with ionic strength of 128 mM and pH 7.9.[3] We recreated similar conditions with our custom LE buffer. The RPA reaction in the membrane used the custom LE buffer with increased primer and probe concentrations compared to the tube-assay. In Figure S-2, we show the RPA reaction taking place within the Millipore glass fiber membrane. The strip was heated to 40°C using a temperature-controlled hot plate for 33 minutes. The positive experiment amplifies more rapidly and provides a significantly higher fluorescence signal than the negative. The negative slightly increases in fluorescence, likely due to non-specific probe interactions.

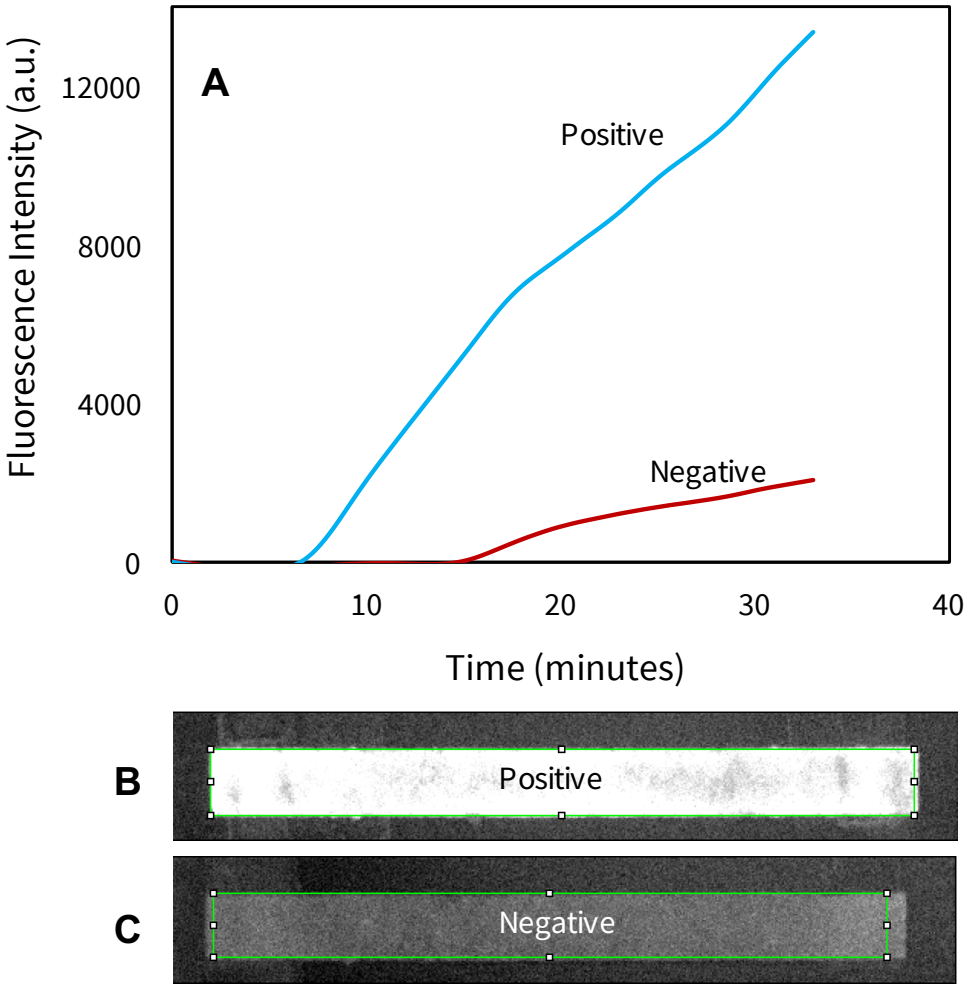

Figure S-2. RPA performed in Millipore glass fiber using a custom LE solution with elevated primer and probe concentrations with respect to the standard tube-based assay. (A) Integrated fluorescence intensity curves of positive (contains target DNA) and negative (no target DNA) amplification reactions on glass fiber substrates as a function of time. The positive reaction (B) begins amplification around approximately 10 minutes and increases in intensity until the end of the experiment. The negative control (C) shows a minimal increase in fluorescence.

Current profiles in simultaneous ITP-RPA

We operated ITP under constant voltage conditions of 150 V with a compliance of 3.5 mA. The typical current traces for simultaneous ITP-RPA from serum (both positive and negative samples)

are shown in Figure S-3. For all experiments, the current remains constant at 3.5 mA for the first 5 minutes and then decreases, roughly exponentially, to a value of 1 mA. The initial voltage is typically 100 V and quickly reduces to around 75 V. After approximately 4 minutes, the voltage rapidly increases to 150 V where ITP conditions transition to constant voltage for the remainder of the diagnostic test. We observed that current and voltage profiles are relatively similar for all experiments. In addition, we observed simultaneous ITP-RPA experiments run in pure buffer versus serum have similar current traces, both in profile and in magnitude.

Most ITP systems employ either constant current or constant voltage conditions. The ITP-RPA assay uses constant current followed by constant voltage in order to control the ITP migration dynamics and optimize Joule heating. The amplification reaction takes place near the center of the strip, after the ITP plug migrates past the serum and proteinase K on the sample pad. We expedite this process with high initial current (3.5 mA), so amplification begins shortly after 5 minutes into the experiment. We then retard the migration so amplification in the ITP plug can continue for another 10 minutes on the strip before eluting into the LE reservoir. Constant voltage conditions slow the migration of the plug with respect to distance along the glass fiber strip. We observed that compliances higher than 3.5 mA resulted in high temperatures in the adjusted TE region, which inhibited RPA and caused accentuated evaporation issues.

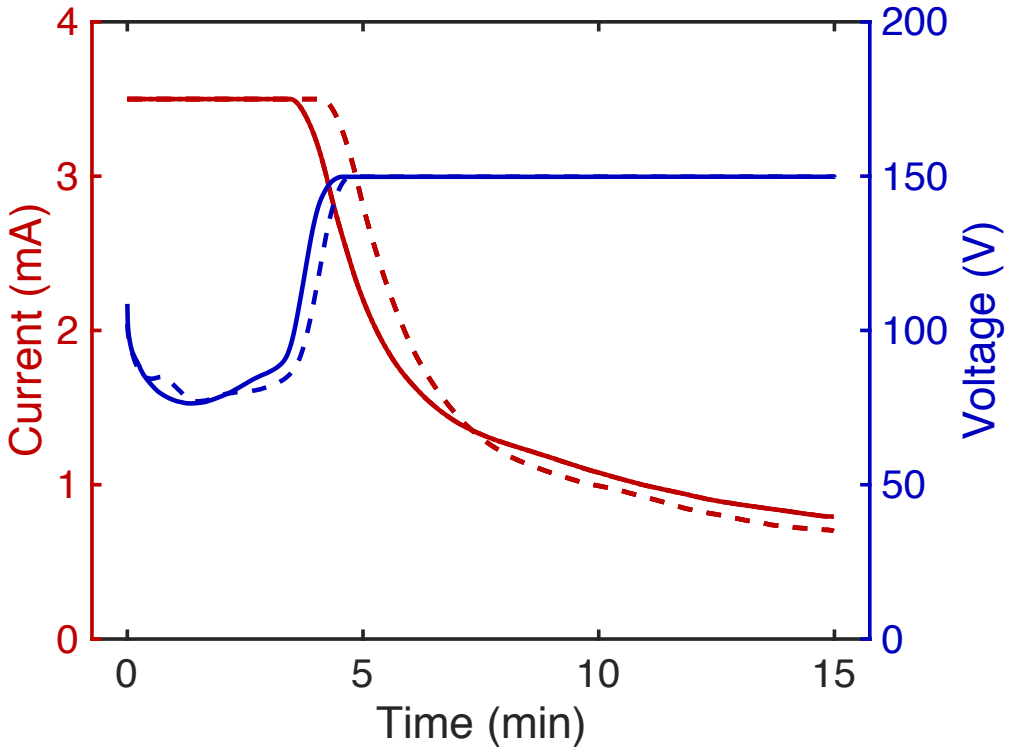

Figure S-3. Typical current and voltage traces of simultaneous ITP-RPA experiments. Experiments were run at a constant voltage of 150 V capped with a 3.5 mA compliance. Here we plot the average current (red) and voltage (blue) for duplicate positive (solid lines) and duplicate negative (dashed lines) experiments.

Joule heating

Figure S-3 shows that significant current passes through the wet glass fiber substrate during simultaneous ITP-RPA reactions. This current can lead to a significant Joule heating and increase in liquid temperature if the generated heat is not properly dissipated from the system.[4,5] The internal temperature rise from Joule heating could be leveraged to provide heat for the RPA reaction. We used infrared (IR) thermal imaging with the iPhone Flir© camera attachment to approximate the spatial and temporal variation of heating due to electrical current application during simultaneous ITP-RPA. Figure S-4 shows thermography images of the device during experiments where the voltage is set to 100 V or 150 V. The images and temperatures reported are not calibrated for the device emissivity and should only be interpreted qualitatively. Under these conditions, the average temperature of the ITP plug as it migrates across the paper strip is approximately 30–34ºC for 100 V and 40–42ºC for 200 V. Different temperature ranges can be

achieved depending on the applied electric field strength, the composition of the ITP electrolytes, and the dimensions of the glass fiber strip. Joule heating is most pronounced in the region of the strip filled with TE, due to its low conductivity. As the ITP plug migrates, the TE region with elevated temperatures covers an increasingly larger area of the glass fiber. The LE portion heats to a lower temperature due to the high conductivity of the electrolyte. Current work in the lab is focused on using solely Joule heating to control the temperature for RPA, eliminating the need for an external heat source.

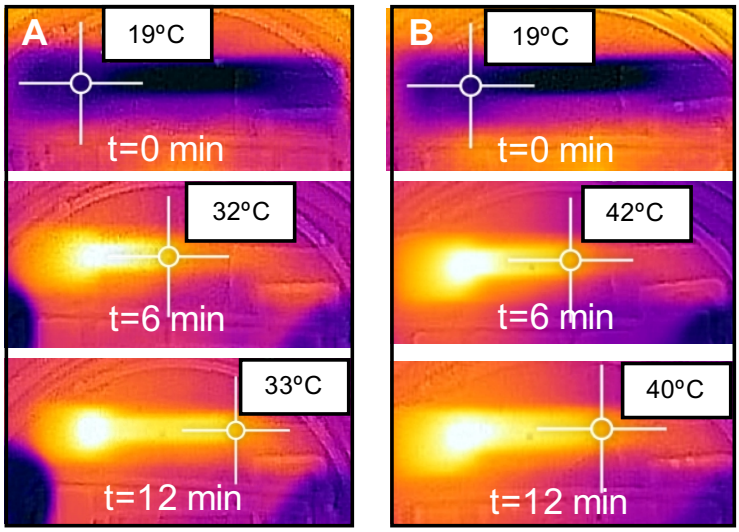

Figure S-4. Thermography images showing the approximate temperature distribution of the substrate due to Joule heating. The temperature is dependent upon the electric field strength, electrolyte solution composition, and the substrate geometry. Due to the conductivity difference between the TE and LE electrolytes, the substrate experiences non-uniform heating. The location of the ITP plug was tracked using a colorimetric dye and the approximate temperatures of the ITP zone on the corresponding IR thermal images are shown. We have used the internal heat generation to provide the required heating for RPA reactions (38–41ºC). The IR images from (A) show a reaction with 100 V applied, while the IR images from (B) show an identical reaction with 150 V applied. The higher electric field in (B) results in a larger temperature rise than in the experiment for (A).

Data Analysis Algorithm for RPA Reactions

Fluorescence intensity emanating from the ITP plug indicates successful amplification of target DNA. We collected a grayscale image of the fluorescence from simultaneous ITP-RPA at each second during the course of the reaction (900 seconds for buffer and serum experiments and 780 seconds for whole blood experiments). We developed a MATLAB algorithm to extract RPA amplification curves from the fluorescence images. The algorithm y-averages the intensity of the image, calculates and subtract the background, sets a threshold, and integrates the intensity that exceeds the threshold. Figure S-5 illustrates this analysis technique for the sample time point of 10 minutes in a positive (1000 cp/rxn) simultaneous ITP-RPA experiment from buffer. The image in Figure S-5A is y-averaged to create a one-dimensional fluorescence intensity profile with respect to distance across the strip, shown in Figure S-5C. Additionally, this was performed at the initial time point preceding ITP initiation (t = 0 min) and averaged over 30 seconds, creating a background as shown in Figure S-5B. This background captured autofluorescence from the glass fiber strip and RPA reagents concentrated near the center of the strip. The background profile was subtracted from each subsequent image to create background subtracted profiles, as shown in Figure S-5D. For creating amplification curves, we then set a threshold of 114% of the average background signal to exclude non-fluorescent signal from negative experiments (see dashed line in Figure S-5D). For determining the LoD, we used a threshold of 50% of the average background signal. We integrated the fluorescence signal that exceeds the threshold to determine the intensity value for each experimental time point. For this sample experiment, the bulk intensity with respect to time is plotted in Figure S-5E.

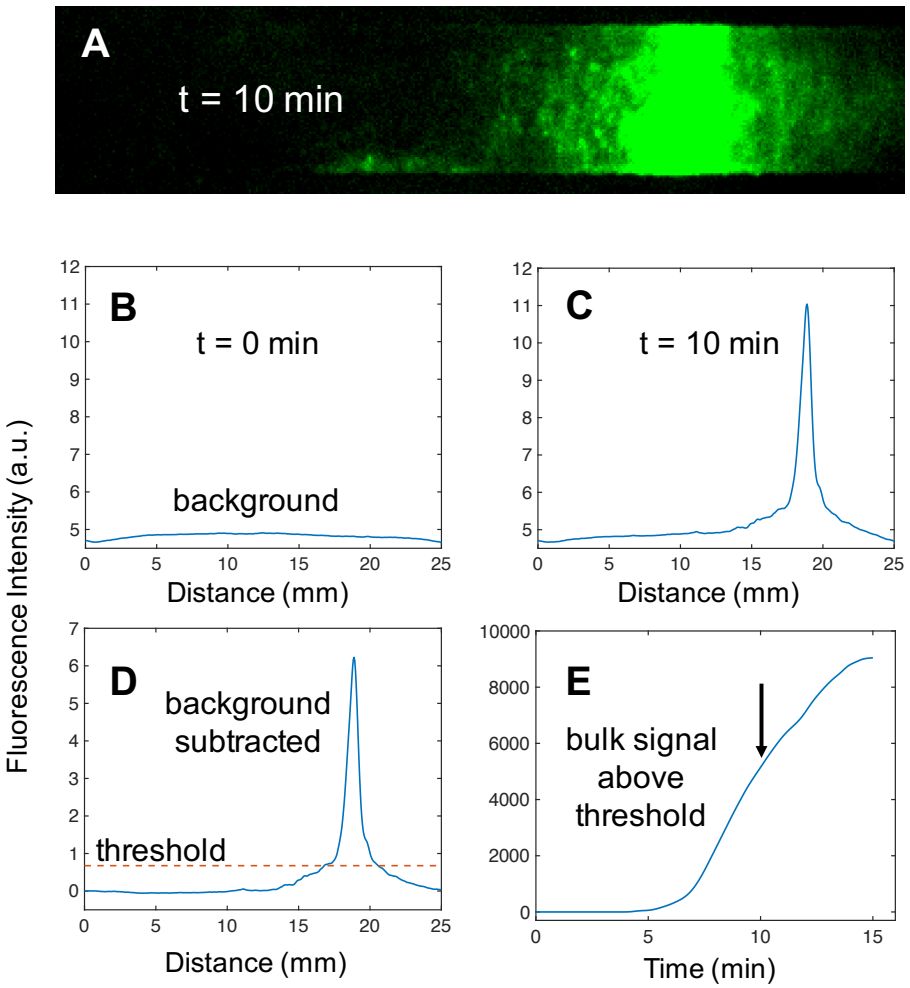

Figure S-5. Data analysis technique for simultaneous ITP-RPA. We illustrate our methodology on a typical positive experiment. (A) Fluorescence images of the reaction are collected over the course of an experiment. Here we show a sample image at the 10 minute time point. (B) The initial images from the experiment are y-averaged to create a background fluorescence profile. (C) The intensity profile at the 10 minute mark shows signal emanating from the reaction within the ITP plug. Regions of the glass fiber strip far from the plug (e.g. 0 to 10 mm) have similar fluorescence to the background. (D) The background is subtracted from all fluorescence profiles in the experiment. A threshold (orange dashed line) is imposed at 114% of the average background intensity. We integrate the signal that exceeds the threshold to find a single fluorescence value for the 10 minute time point. (E) The arrow indicates the fluorescence value at 10 minutes within the amplification curve of the experiment.

Simultaneous ITP-RPA with human serum samples

In Figure 5, we show amplification curves and semi-quantitative data from the simultaneous ITP-RPA assay with serum samples. Here we show fluorescence images of the integrated ITP-RPA reaction over a 15 minute timespan in Figure S-6A. The serum increases the amount of trailing fluorescence on the glass fiber when compared to ITP-RPA with pure buffer shown in Figure 3A. As discussed in the manuscript, this may be due to undigested serum proteins forming complexes with DNA or fluorophores. These complexes have slow electrophoretic mobilities that prevent them from focusing in the ITP plug. Between 5 and 10 minutes, the RPA reaction proceeds and causes fluorophores from the probe to fluoresce within the ITP plug. Figure S-6B shows a spatiotemporal map of this reaction, exhibiting ITP migration dynamics similar to experiments run in pure buffer (Figure 3A). The map shows a differing velocity profile of the ITP plug, which is halted for several minutes (from the 5 to 10 minute marks) near the center of the strip. Observations from the experiment revealed that pronounced electroosmotic counterflow contributed to stalling the plug velocity. The plug increases in width as the endpoint fluorescence magnitude increases (common at high input copy numbers).

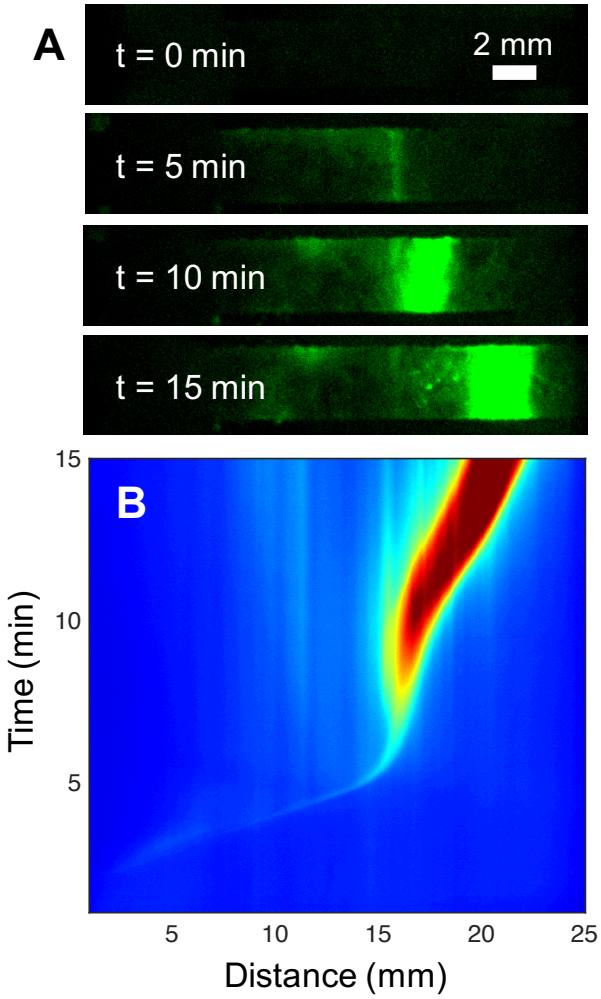

Figure S-6. Visualization of the migration and amplification dynamics of simultaneous ITP-RPA from serum samples. (A) Experimental fluorescence images of a positive integrated ITP-RPA reaction (10^5 cp/mL) on glass fiber from human serum. (B) Spatiotemporal intensity map of the reaction and ITP dynamics as they progress.

To provide a full representation of our assay's quantitative reproducibility, we provide real-time normalized integrated fluorescence values for all replicates at the respective concentrations of target DNA in human serum (see Figure S-7). This plot provides supplementary information to that of Figure 5A, which only shows average real-time integrated fluorescence.

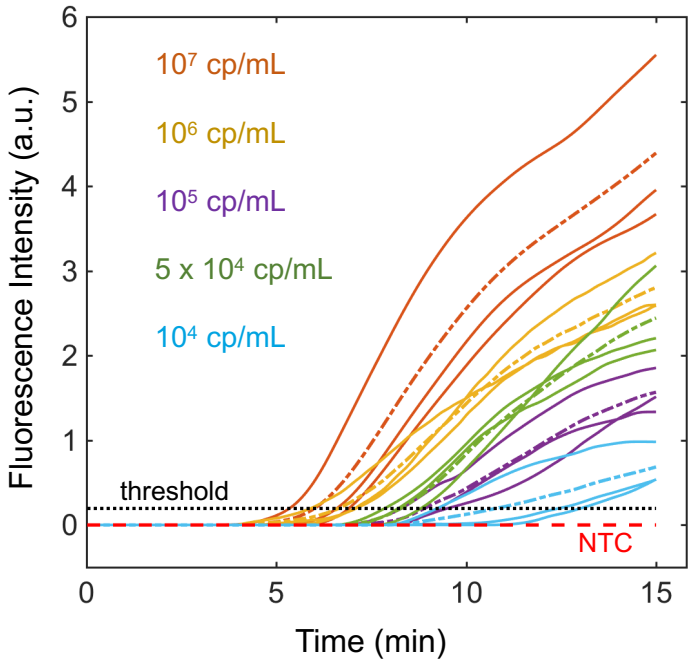

Figure S-7. Fluorescence intensities from experiments during simultaneous DNA extraction and RPA amplification in human serum spiked with target DNA. (A) Integrated RPA fluorescence intensities plotted with respect to time for a dilution series of DNA concentrations. All replicates (N=3) are shown here in contrast to Figure 5A which plots the average intensity profiles for each respective concentration.

Limit of detection of simultaneous ITP-RPA

The limit of detection (LoD) of our assay (10^4 cp/mL of serum) was rigorously defined using the CLSI recommendations for *in vitro* diagnostics. This definition is appropriate when measuring signal output from an instrument, such as fluorescence intensity in some microscopy applications. Figure S-8 illustrates how we determined the LoD of the simultaneous ITP-RPA assay in human serum. In Figure S-8A, we plot integrated fluorescence intensities for the two lowest concentrations we could detect. For the LoD analysis, the threshold of the custom analysis algorithm was set at 50% of the average background intensity (see Figure S-5). The endpoint fluorescence signal intensity for each replicate determined the LoD of the assay. In Figure S-8B, we show the average endpoint fluorescence intensity with a 95% confidence interval. The confidence interval of the assay at 10^4 cp/mL does not overlap with variations of the NTC signal (LoB). Therefore at this concentration, we can detect target DNA with 95% probability, and we define our LoD to be 10^4 cp/mL of serum.

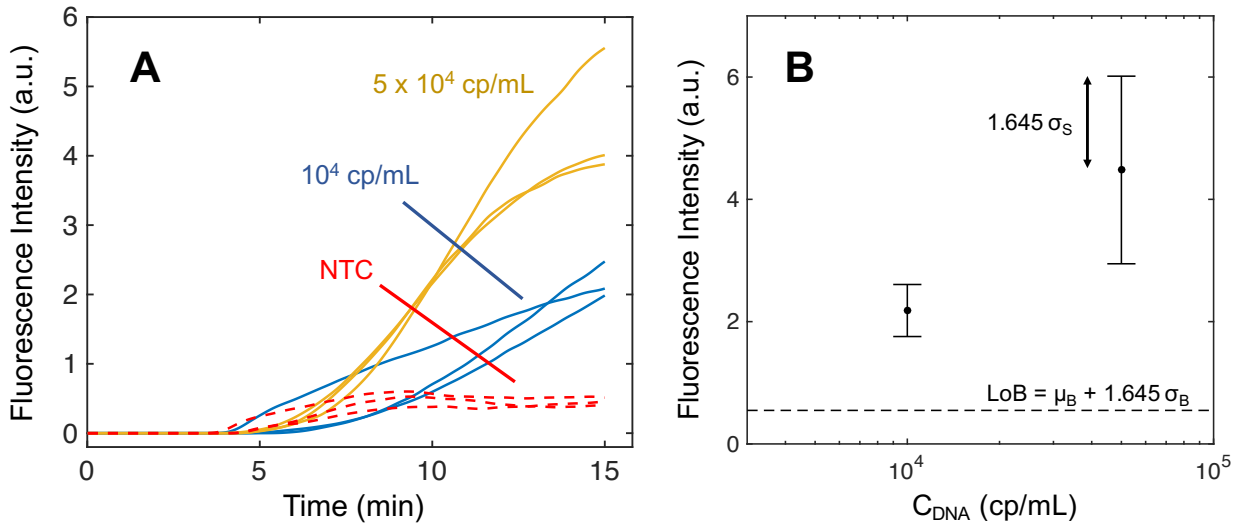

Figure S-8. Limit of detection (LoD) analysis of simultaneous ITP-RPA from human serum samples. (A) Fluorescence intensity profiles resulting from amplification at low concentrations of input DNA. (B) Visual representation of the LoD. The dashed line is the limit of blank (LoB). The error bars represent 1.645 times the standard deviation of the sample at the respective concentration. The LoD was determined to be 10^4 cp/mL as its average fluorescence and corresponding error are above the LoB.

Supplemental data for simultaneous ITP-RPA in pure buffer

In Figure S-9, we provide the individual amplification curves for three trials for the data provided in Figure 4. We also include negative control trials with foreign DNA (shear salmon sperm DNA) to verify simultaneous ITP-RPA does not affect the high specificity of RPA. The test consistently and accurately reports each experiment with target DNA as positive (above the NTC) although there is significant quantitative variation across the three trials at these relatively low copy number experiments. These low copy number experiments represent concentrations of target that are significantly less than those relevant to HIV viral load testing (10^3 cp/mL to 10^7 cp/mL). If one were using this diagnostic at these low target concentrations, it would be used as qualitative test (target present or not present) and thus quantitative reproducibility is not the primary concern here.

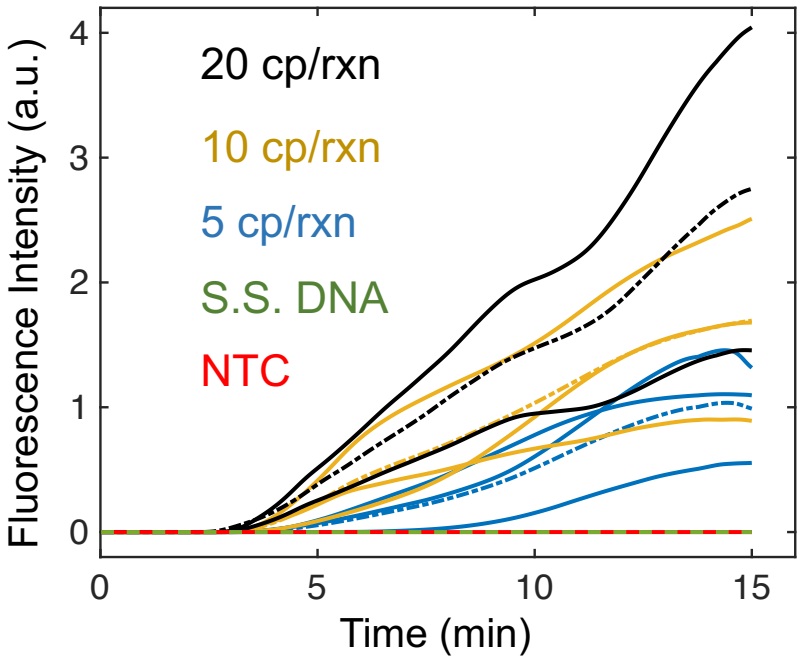

Figure S-9. Simultaneous ITP-RPA experiments from pure buffer were analyzed to generate fluorescence curves with respect to time for low input DNA copy numbers. We present individual trials for 20 cp/rxn (N=2), 10 cp/rxn (N=3), 5 cp/rxn (N=3), no template control (NTC) (N=3), and 10 pg sheared salmon sperm DNA (N=2). The custom analysis algorithm used a threshold of 114% of the average background signal, so nonspecific signal from the negative controls was negligible.

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